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PROVISIONAL APPLICATION FOR PATENT COVER SHEET

This is a request for filing a **PROVISIONAL APPLICATION FOR PATENT** under 37 C.F.R. § 1.53(c)

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TITLE OF INVENTION (280 characters max)

BILIVERDIN DETECTION ASSAY FOR USE IN BIRDS AND REPTILES

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ENCLOSED APPLICATION PARTS (*Check All That Apply*)

- ☒ Provisional Application Cover Sheet — *Number of pages* [2]
- ☒ Specification — *Number of pages* [16]
- ☒ Claims — *Number of pages* [1]
- ☐ Abstract — *Number of pages* [ ]
- ☐ Drawing(s) — *Number of Sheets* [ ]
- ☐ Small Entity
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- ☐ Assignment
- ☒ Other (Specify): Authorization to Treat Reply Requiring Extension of Time as Incorporating Petition for Extension of Time

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The invention was made by an agency of the United States Government or under a contract with an agency of the United States Government.

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Respectfully submitted,

SIGNATURE: Gwendolyn D. Spratt **Date** 8/27/02

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CERTIFICATE OF EXPRESS MAILING UNDER 37 C.F.R. § 1.10

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PATENT

PROVISIONAL PATENT APPLICATION

TO ALL WHOM IT MAY CONCERN:

Be it known that we, Christopher Gregory and Branson W. Ritchie, citizens of the United States of America, residing respectively at 1015 Cooper Farm Road, Nicholson, GA 30565 and 1080 Barnett Place, Athens, GA 30605, U.S.A., have invented new and useful improvements in

BILIVERDIN DETECTION ASSAY FOR USE IN BIRDS AND REPTILES

for which the following is a specification.

ASSAYS FOR THE DETECTION OF BILIVERDIN IN BIRDS AND REPTILES

FIELD OF THE INVENTION

The present invention relates to assays for the detection of bililverdin in birds and reptiles.

BACKGROUND OF THE INVENTION

Birds and reptiles commonly are presented for clinical illness related to liver disease. Biochemical tests, such as serum enzyme activities and bile acid concentration, have been used for the antemortem diagnosis of liver disease in birds and reptiles, but these tests have clinical limitations. In mammals, bilirubin quantitation is used to assess increased erythrocytic destruction and cholestasis. Hyperbilirubinemia is often observed in liver and hemolytic disease. Therefore, total, conjugated, and unconjugated serum bilirubin concentrations and the presence or absence of bilirubinuria are used routinely in mammals as screening tests for hepatic function or extrahepatic disease. Liver diseases associated with hepatocellular swelling, hepatic fibrosis, hepatic inflammation or extrahepatic diseases resulting in red cell hemolysis, bile duct inflammation or obstruction may cause hyperbilirubinuria and bilirubinuria. The measurement of conjugated bilirubin in urine is of value in mammals because bilirubinuria often precedes the onset of hyperbilirubinemia. Measuring the serum concentrations of conjugated and unconjugated bilirubin can aid in differentiating extrahepatic from intrahepatic disease in mammals.

Birds and reptiles lack biliverdin reductase, the enzyme responsible for conversion of biliverdin to bilirubin; therefore, they do not produce bilirubin. In birds and reptiles, biliverdin (not bilirubin) is the waste product of hemoglobin destruction. Since bilirubin is not usually present in avian and reptile serum and urine, its measurement is not useful in evaluating liver disease or hemolytic disease in birds. The majority of biliverdin is excreted in an unconjugated form into the bile. Blood biliverdin

concentration increases when hepatic regurgitation due to decreased function or biliary obstruction occurs, resulting in the typical green discoloration of plasma or urine.

The present invention provides a biochemical assay to quantitate the biliverdin present in plasma, serum or biological fluids collected from birds and reptiles. This test will prove of benefit as an adjunct to serum enzyme activities and bile acid concentrations in evaluating intra- and extrahepatic diseases in birds and reptiles.

SUMMARY OF THE INVENTION

The present invention provides a method of measuring increased biliverdin concentration in a sample from an avian or reptilian species comprising: a) contacting the sample with biliverdin reductase; and b) measuring a change in absorbance at 450nm, wherein an increase in absorbance as compared to a control sample indicates increased biliverdin concentration in the sample.

Also provided by the present invention is a method of determining biliverdin concentration in a sample from an avian or reptilian species comprising: a) contacting the sample with biliverdin reductase; b)measuring a change in absorbance at 450nm; and c) determining biliverdin concentration by comparing the absorbance value obtained in step b) with absorbance values on a standard concentration curve.

DETAILED DESCRIPTION OF THE INVENTION

The present invention may be understood more readily by reference to the following detailed description of the preferred embodiments of the invention and the Example included herein.

Before the present compounds and methods are disclosed and described, it is to be understood that this invention is not limited to specific methods, as such may, of course,

vary. It is also to be understood that the terminology used herein is for the purpose of describing particular embodiments only and is not intended to be limiting.

It must be noted that, as used in the specification and the appended claims, the singular forms "a," "an," and "the" include plural referents unless the context clearly dictates otherwise.

The present invention provides a method of measuring increased biliverdin concentration in a sample from an avian or reptilian species comprising: a) contacting the sample with biliverdin reductase; and b) measuring a change in absorbance at about 450nm, wherein an increase in absorbance as compared to a control sample indicates increased biliverdin concentration in the sample.

The samples of the present invention can be obtained directly from an avian or reptilian species or purchased from other sources. One of skill in the art can utilize the methods of the present invention to analyze samples that are obtained by the skilled artisan or to analyze samples that are obtained by other parties and later provided to the skilled artisan.

The avian species from which samples can be obtained include, but are not limited to, *Tinamiformes* (tinamous), *Struthioniformes* (ostrich and allies), *Podicipediformes* (grebes), *Sphenisciformes* (penguins), *Procellariiformes* (albatrosses and fulmars), *Pelecaniformes* (cormorants and pelicans), *Anseriformes* (waterfowl, e.g. ducks, geese, swans), *Phoenicopteriformes* (flamingos), *Ciconiiformes* (herons, storks, New World vultures and relatives), *Falconiformes* (hawks and vultures), *Galliformes* (domestic fowl and game birds), *Gruiformes* (cranes, hemipodes, gallinules), *Charadriiformes* (gulls and shore birds), *Gaviiformes* (divers, loons), *Columbiformes* (doves and pigeons), *Psittaciformes* (parrots), *Coliiformes* (mousebirds), *Musophagiformes* (turacos), *Cuculiformes* (cuckoos), *Strigiformes* (owls), *Caprimulgiformes* (goatsuckers), *Apodiformes* (swifts and hummingbirds), *Trogoniformes*

(trogons), *Coraciiformes* (rollers, kingfishers and relatives), *Piciformes* (woodpeckers, toucans and relatives), *Passeriformes* (songbirds, or passerines and perching birds)

The reptiles from which samples can be obtained include, but are not limited to, *Crocodylidae*(Caiman and Crocodiles), *Squamata* Suborder *Amphisbaenia* (Worm-Lizards), *Amphisbaenidae* Family (Worm-Lizards), Suborder *Sauria* (Lacertilia) (Lizards), *Anguidae* Family (Alligator Lizards), *Corytophanidae* Family (Basilisks), *Gekkonidae* Family (Geckos), *Iguanidae* Family (Iguanas),*Phrynosomatidae* Family (Spiny Lizards), *Polychrotidae* Family (Anoles), *Scincidae* Family (Skinks), *Teiidae* Family (Whip-tailed Lizards), *Xantusiidae* Family (Night Lizards), Suborder *Serpentes* (Ophidia) (Snakes), *Anomalepididae* Family (Dawn Blind Snakes), *Boidae* Family (Boas), *Colubridae* Family (Harmless Snakes), *Elapidae* Family (Coral Snakes), *Hydrophiidae* Family (Sea Snakes), *Leptotyphlopidae* Family (Thread Snakes), *Viperidae* Family (Vipers), Order *Testudinata* (Chelonia) (Turtles and Tortoises), Suborder *Cryptodira* (Hidden-Necked Turtles),*Cheloniidae* Family (Sea Turtles), *Chelydridae* Family (Snapping Turtles), *Deromchelyidae* Family (Leatherback Sea Turtle), *Emydidae* Family (Pond and River Turtles), *Kinosternidae* Family (Mud and Musk Turtles), *Testudinidae* Family (Tortoises).

The sample of this invention can be from any bird or reptile and can be, but is not limited to, plasma, serum, urine, peripheral blood, sputum, fine needle aspirations or other biological fluid.

As mentioned above, bilirubin reductase is an enzyme responsible for the conversion of biliverdin to bilirubin. Therefore, by contacting biological samples with biliverdin reductase, any biliverdin present in the sample should be converted to bilirubin, thus allowing one of skill in the art to measure the amount of biliverdin present in the sample by measuring the change in absorbance at about 450 nm that results from bilirubin production. Biliverdin reductase can be obtained from commercial sources such as ICN Biochemicals or the enzyme can be cloned and produced by standard recombinant methods as described in the Examples herein. The biliverdin reductase can be from rat,

mouse, human or other mammalian origin. One of skill in the art can determine the specific activity of a recombinant biliverdin reductase preparation as well as the concentration of biliverdin reductase necessary for conversion of biliverdin to bilirubin. The preparation can be such that no other bacterial proteins or other contaminants are present in the preparation or the preparation can be purified such that all or most bacterial proteins are removed.

The contacting step of the methods described herein can be performed in any order. For example, biliverdin reductase can be added to a sample in a microcuvette or other sample container, or the sample can be added to biliverdin reductase present in a microcuvette or other sample container.

By utilizing the methods of the present invention, one of skill in the art can measure increased biliverdin concentration in a particular bird or reptile. For example, a sample can be obtained from a bird or reptile. This sample is then contacted with biliverdin reductase and a change in absorbance (absorbance/minute) is measured as described in the Examples. This change in absorbance is compared with the change in absorbance in a control sample. The control sample can be a sample from a bird or reptile with normal levels of biliverdin. One of skill in the art can utilize the methods of the present invention to determine normal levels or a range of biliverdin concentration corresponding to normal levels in a particular avian or reptilian species. These values can then be compared to biliverdin concentration in test samples to determine whether or not there is increased biliverdin concentration or an amount of biliverdin corresponding to a disease state. Once the skilled artisan compares the change in absorbance in the sample with the control sample, if the change in absorbance is greater in the sample, the sample contains increased or elevated levels of biliverdin as compared to the biliverdin levels in a normal, non-diseased bird or reptile. These increased levels of biliverdin could indicate the presence of a disease state. In the methods of the present invention, any change in absorbance in a control sample that is not contacted with the biliverdin reductase can also be measured. Any change in absorbance observed with this sample

can be subtracted from test values in order to eliminate background absorption unrelated to biliverdin reductase activity.

One of skill in the art could also utilize this method to monitor a particular bird or reptile. For example, once a bird or reptile has been identified as having increased biliverdin levels, samples can be obtained from the same bird or reptile in the future and compared with previous samples from the same bird or reptile in order to determine the progression of the disease. For example, a sample obtained at a later date can be compared with a sample obtained previously. If the change in absorbance is greater in the sample obtained at a later date as compared with the change in absorbance associated with the previous sample, this would indicate that biliverdin levels are continuing to increase in the bird or reptile. Similarly, if a change in absorbance is less in the sample obtained at a later date as compared with the change in absorbance associated with the previous sample, this would indicate that biliverdin levels are decreasing in the bird or reptile. By utilizing these comparative studies, one of skill in the art can monitor drug efficacy as well.

The disease states that can be diagnosed and prognosed utilizing the methods of the present invention include, but are not limited to, liver diseases such as hepatitis and those associated with hepatocellular swelling, hepatic fibrosis and/or hepatic inflammation. The disease states can be infectious (i.e. bacterial, viral, chlamydial, fungal, mycoplasmal or parasitic) as well as noninfectious (i.e., metabolic, circulatory, neoplastic, traumatic, nutritional, biochemical or toxic). The methods of the present invention can also be utilized to diagnose and/or prognose nonhepatic disease such as hematologic diseases (for example, hemolytic disease, both extravascular and intravascular or other inherited/acquired conditions that result in increased erythrocyte destruction and hemoglobin degradation). Extrahepatic diseases such biliary tract occlusion resulting in decreased excretion of biliverdin via the biliary tract into the intestine can also be diagnosed and/or prognosed utilizing the methods of the present invention.

The present invention also provides a method of determining biliverdin concentration in a sample from an avian or reptilian species comprising: a) contacting the sample with biliverdin reductase; b) measuring a change in absorbance at about 450nm; and; c) determining biliverdin concentration by comparing the absorbance value obtained in step b) with absorbance values on a standard concentration curve.

In addition to measuring relative increases or decreases in biliverdin concentration, the present invention allows the determination of biliverdin concentrations in biological samples obtained from birds and reptiles. Upon measuring the change in absorbance for a particular sample, one of skill in the art can determine the concentration of biliverdin in the sample by calculating a standard curve of concentration (y-axis) vs. change in absorbance (x-axis), determining the "best fit" linear regression equation and using this equation to measure biliverdin concentrations in biological samples from birds and reptiles. As described in the Examples, the change in absorption is calculated over a time period. For example, the change in absorption can be measured between about 0.25 and about 1.25 minutes, between about 0.3 minutes and about 1.3 minutes, between about 0.40 minutes and about 1.4 minutes, between about 0.5 minutes and about 1.5 minutes. Other time periods are also contemplated by this invention as long as an accurate measure of biliverdin concentration results from the time period chosen. Based on the data provided herein, normal serum values appear to be about <0.5 mg/dl. Therefore, sample concentrations greater than about 0.5 mg/dL may indicate a disease state. Table 1 shows the values of a standard curve utilized to determine biliverdin concentrations as well as the concentration values observed for several test samples.

By measuring biliverdin concentrations, a particular bird or reptile can be monitored. For example, if a sample yields a biliverdin concentration of 0.7 mg/dL and at a later time, a sample from the same bird or reptile yields a biliverdin concentration of 0.9 mg/dL, one of skill in the art would know that the disease had progressed. Alternatively, if a sample yields a biliverdin concentration of 0.7 mg/dL and at a later time, a sample from the same bird or reptile yields a biliverdin concentration of 0.4 mg/dL, one of skill in the art would know that the bird's condition had improved. These

comparative methods can also be utilized to determine drug efficacy. By monitoring increases or decreases in biliverdin concentration after the administration of a drug, medication or therapy, the efficacy of a particular drug, medication or therapy can be determined.

One of skill in the art could obtain numerous samples from a number of different bird and reptile species in order to create ranges of biliverdin concentration values that can be correlated to a particular disease state. One skilled in the art will then be able to correlate the levels of biliverdin detected using the methods disclosed herein with a particular stage of disease, thus utilizing the detection method for prognostic purposes. The prognostic evaluation can determine what type of drug or medication to administer at different stages of disease depending on the amounts of biliverdin detected in the sample.

All of the methods described herein can be used in combination with other methods utilized to detect serum enzyme activities and bile acid concentrations to provide a panel of results that will aid in the diagnosis and prognosis of disease. These methods can also be utilized in conjunction with visual inspection of a biological sample from a bird or reptile which can be pigmented due to the presence of biliverdin.

By utilizing the methods of the present invention, one of skill in the art could monitor the conversion of biliverdin to bilirubin over a time period and determine a particular time point in which the absorbance value at 450 nm would be an accurate measure of the amount of bilirubin present in the sample. In this way, one of skill in the art could obtain absorbance values at a certain time after contacting the sample with bilirubin reductase and correlate it to a particular concentration of bilirubin.

In addition to the above-described methods, there are other assays that can be utilized to measure biliverdin levels in biological fluids. One of these involves using the basic assay of body fluids in which biliverdin is converted to bilirubin via biliverdin reductase in the presence of NADH or NADPH. In this method, the skilled artisan can measure the change in absorbance (typically at about 340 nm) as NADH or NADPH is

converted to NAD. Biliverdin concentrations can then be determined as described herein and in the Examples, via the construction of a standard curve and subsequent comparison of the change in absorbance for the test sample with the values on the standard curve.

Since these assays are based on the conversion of biliverdin to bilirubin, one of skill in the art could also utilize commercially available reagents to measure converted biliverdin (i.e., newly formed bilirubin). These methods include urine and serum reagent strip/pad (dipstick) methodology and reagent kits based on the diazo method. The diazo method is described in Winsten and Cehelyk ("A rapid diazo technique for measuring total bilirubin," *Clin Chim. Acta* 25: 441 (1969)) and in Walters and Gerarde ("An ultramicromethod for the determination of conjugated and total bilirubin in serum or plasma," Microchem. J. 15:231 (1970)), both of which incorporated herein in their entireties by this reference.

The present invention further provides a kit for the detection of biliverdin in biological samples from birds or reptiles comprising: a) recombinant biliverdin reductase enzyme; b) potassium phosphate buffer; c) NADH; and d) biliverdin.

The following examples are put forth so as to provide those of ordinary skill in the art with a complete disclosure and description of how the compounds, compositions, articles, devices and/or methods claimed herein are made and evaluated, and are intended to be purely exemplary of the invention and are not intended to limit the scope of what the inventors regard as their invention.

EXAMPLES

Recombinant rat biliverdin reductase

A. RNA Extraction and Amplification

Primers were designed to amplify a 969 base pair (bp) DNA product using rat biliverdin reductase sequence available from GenBank (accession no. NM_053850 and based on Ennis et al.). The sense primer, KPNBILIVF, was designed to contain a KPN I

restriction site (underlined). The antisense primer, XBABILIVR, was designed to contain an XBA I restriction site (underlined).

KPNBILIVF

5′-GGGGTACCCCGACTGAAGAGACCGCCATGGATGCCGAGC-3′

XBABILIVR

5′-GCTCTAGAGCGACATGTTCCATGGTAAGAGCTAAAGGTGA-3′

Mechanical disruption of rat liver and kidney tissue was performed using a Micro-mincer (BioSpec Products, Bartlesville, OK)-a comparable apparatus may be used.

Minced tissue was further homogenized with a QIAshredder (Qiagen, Inc. Valencia, CA).

RNA was extracted from the disrupted, homogenized tissue with an RNeasy Mini Kit (Qiagen, Inc. Valencia, CA) per manufacturer's instructions. Comparable methods also may be used.

A commercially available kit was used for RT-PCR (Titan One Tube RT-PCR Kit, Roche Molecular Biochemicals, Indianapolis, IN); comparable methods may be used. 1 µl (approximately. 1 ug/ul) of the extracted RNA is added to a 49 µl RT-PCR working solution containing:

29.5 µl of DEPC-treated H_2O
200 µM dNTPs (4 µl of 10 mM dNTP mix) (included in kit)
2 µM of each of the sense and antisense primers (1 µl of a 10 pmol/µl primer solution)
5 mM DTT solution (2.5 µl of 100 mM solution) (included in kit)
10 µl of 5X Buffer (included in kit)

1 ul of enzyme mix (included in kit)

Cycling Parameters:

Reverse transcription:

1 cycle of 42C for 30 min

Denaturation of RNA/cDNA hybrid:

1 cycle of 94C for 2 min

Amplification:

1 cycle of 94C for 2 min

5 cycles of:

94C for 1 min

50C for 2 min

68C for 3 min

35 cycles of:

94C for 30 sec

55C for 30 sec

68C for 3 min

1 cycle of 68C for 10 min

Analysis of PCR reactions

The amplicon was analyzed on a 1% agarose gel containing a 1:10,000 dilution of SYBR Gold nucleic acid gel stain (Molecular Probes, Eugene, OR).

Five µL PCR product plus 2 µL loading buffer was added to each well. The gel was transilluminated and visualized with UV light.

B. Cloning and expression of recombinant rat biliverdin reductase

The 969 bp amplicon was excised from the gel and extracted from the agarose using a GenElute Agarose Spin Column (Sigma Chemical Co., St. Louis, MO). The DNA was precipitated by the addition of 2X volume of ethanol, 0.1X 3M sodium acetate, and freezing at –70 C for 30 minutes. After centrifugation for 20 minutes at 12000 rpm, the pellet was washed in 500 ul 70% ethanol, centrifuged for 10 minutes at 12,000 rpm, and air dried. The DNA subsequently was re-dissolved in 100 ul of DEPC-treated deionized sterile water to an approximate concentration of 1ug/ul). 10 ul aliquots of the amplicon were added to tubes containing 1 unit each of KPN I and XBA I (Roche Molecular Biochemicals, Indianapolis, IN). In addition, the expression vector pBAD/gIII B (Invitrogen, Carlsbad, CA) also was added to a KPN I/ XBA I mixture and both samples were incubated at 37°C for one hour and then electrophoresed on a 1% agarose gel containing 1/10,000 concentration of Sybr Gold. Bands were excised and processed as described above. Both products were reconstituted in 20 ul DEPC-treated deionized sterile water, approximate concentration 1 ug/ul. 10 ul of each solution was added to a 50 ul solution containing 1 unit T4 DNA ligase and buffer (Roche Molecular Biochemicals, Indianapolis, IN) and incubated for 16 hours at 4°C. Chemically competent *Escherichia coli* (TOP10F', Invitrogen, Carlsbad, CA) was transformed with the ligated pBAD/GIII B/ recombinant rat biliverdin reductase ligation product and recombinant rat biliverdin reductase expression was induced with arabinose with modification of the manufacturer's instructions. The transformed bacteria were grown for 16 hours in 50 ml of LB broth containing 2% arabinose.

Crude purification of the protein was performed by pelleting the bacteria with centrifugation at 6,000 rpm for 10 minutes at 4°C. After decanting the supernatant, the bacterial pellet was resuspended in 1 ml lysis buffer (50 mM NaH2PO4, 300 mM NaCl, 5 mM imidazole, and 50 mM Tris HCl, pH 8.0) and sonicated. The sonicated mixture was centrifuged again at 6,000 rpm and the supernatant was transferred to a new tube.

The enzyme mixture was analyzed by SDS PAGE on a 12% Tris glycine polyacrylamide gel stained with Coomassie Brilliant Blue and detection of an approximately 37.5 kd band. Confirmation that the band was indeed recombinant rat

biliverdin reductase was by Western blotting of a replicate unstained polyacrylamide gel using a commercially available rabbit anti-biliverdin reductase antibody (StressGen Biotechnologies Corp., Victoria, BC Canada). A commercially available recombinant rat biliverdin reductase (StressGen Biotechnologies Corp., Victoria, BC Canada) was used as a positive control. Negative controls were bacteria transformed with pBAD/GIII B plasmid without a DNA insert.

Kinetic assay using recombinant rat biliverdin reductase

The kinetic assay was performed on a Beckman DU-530 spectrophotometer (comparable spectrophotometers may be used). The assay is based on one used in references 2 (Kutty and Mianes) and 3 (Fakhrai and Maines), both of which are incorporated herein by this reference in their entireties. Briefly, the change in absorbance at 450 nm is monitored for 2 minutes at room temperature. The value Δabs/min is determined using the measured interval from 0.25 to 1.25 minutes. A standard curve is used to determine sample concentration.

Construction of a standard curve

Commercially available biliverdin dihydrochloride (BV; ICN Biomedicals Inc., Aurora, OH) is dissolved in 100% DMSO to a concentration of 2.5 g/dl.

Standard concentrations of 0.5, 1.0, 5.0, 10.0, 15.0, and 20.0 mg/dl of BV are made in commercially available chicken serum (Invitrogen, Carlsbad, CA)

Reagents used (modified and/or based on references 1 and 2):

Recombinant rat biliverdin reductase enzyme mix (BVR)

0.1M potassium phosphate buffer, pH 7.0 containing 0.1mg/ml

bovine serum albumin (KP buffer)
5 M NADH (ICN Biomedicals, Aurora, OH)

Kinetic assay:

125 ul of KP buffer is added to 160 ul of HPLC grade water and 37.5 ul of BVR
50 ul of standard or unknown sample is added to the reagent mix
2.5 ul of 5M NADH is added to a microcuvette

The reagent sample mix (372.5 ul) is added to the microcuvette and the sample is IMMEDIATELY blanked and the assay is begun. The standard curve is plotted on an absorbance vs. time standard curve and a regression equation with the highest R2 value is determined. Unknown sample concentrations of biliverdin are determined using the regression equation.

Throughout this application, various publications are referenced. The disclosures of these publications in their entireties are hereby incorporated by reference into this application in order to more fully describe the state of the art to which this invention pertains.

Although the present process has been described with reference to specific details of certain embodiments thereof, it is not intended that such details should be regarded as limitations upon the scope of the invention except as and to the extent that they are included in the accompanying claims.

REFERENCES

1. Ennis O, Maytum R, and Mantle TJ. 1997. Cloning and overexpression of rat kidney biliverdin IXα reductase as a fusion protein with glutathione S-transferase:

stereochemistry of NADH oxidation and evidence that the presence of the glutathione S-transferase domain does not effect BVR-A activity. Biochem J 328:33-36.

2. Kutty RK and Maines MD. 1981. Purification and characterization of biliverdin reductase from rat liver. J Biol Chem 256: 3956-3962.

3. Fakhrai H and Maines MD. 1992. Expression and characterization of a cDNA for rat kidney biliverdin reductase: evidence suggesting the liver and kidney enzymes are the same transcript product. J Biol Chem 267: 4023-4029.

Table 1. Standard curve and assay of "normal" serum

Abs/min [measured]	Sample concentration mg/dl	Reported value
0.0912	15	standard
0.0791	10	standard
0.0472	5	standard
0.0114	1	standard
0.0067	0.5	standard
0.0011	186194 10-12-01	<0.5
0	187059	<0.5
0	loggerhead	<0.5
0.0028	190633 owl	<0.5
0	187129 heparin avian	<0.5
0.0048	172970 turtle	<0.5
0.0003	177753 tortoise	<0.5
0	177754 tortoise	<0.5
0.0002	167718 tortoise	<0.5
0.0013	190615 toucan	<0.5
0.0003	190630 owl	<0.5
0.004	190629 owl	<0.5
0	187053 macaw	<0.5
0.0026	190657 cockatoo	<0.5
0.0011	190628 owl	<0.5
0.001	141247 owl	<0.5
0	181097 lizard	<0.5
0.0331	unknown 2 mg/dl	<0.5
0.0243	unknown 1 mg/dl	<0.5
0.0489	unknown 4 mg/dl	<0.5
0.0535	unknown 6 mg/dl	<0.5
0.0107	unknown 0.2 mg/dl	<0.5
0.0006	unknown 0 mg/dl	<0.5

What is claimed is:

1. A method of measuring increased biliverdin concentration in a sample from an avian or reptilian species comprising:
   a) contacting the sample with biliverdin reductase; and
   b) measuring a change in absorbance at 450nm, wherein an increase in absorbance as compared to a control sample indicates increased biliverdin concentration in the sample.

2. A method of determining biliverdin concentration in a sample from an avian or reptilian species comprising:
   a) contacting the sample with biliverdin reductase;
   b) measuring a change in absorbance at 450nm; and
   c) determining biliverdin concentration by comparing the absorbance value obtained in step b) with absorbance values on a standard concentration curve.